CELL BIOASSAY FOR THE DETECTION OF CIGUATOXINS, BREVETOXINS, AND SAXITOXINS

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We have developed an assay using neuroblastoma cells for detection of sodium channel-specific marine toxins based on an end point determination of mitochondrial dehydrogenase activity in the presence of veratridine and ouabain. This cell bioassay allows detection of either sodium channel blocking agents, such as saxitoxins, or sodium channel enhancers such as brevetoxins and ciguatoxins. The assay responds in a dose dependent manner, differentiates the toxic activity as either sodium channel blocking or enhancing, and is highly sensitive. Assay response to brevetoxins and to ciguatoxic extracts is rapid, allowing dose dependent detection within 4-6hr. The method is simple, utilizes readily available reagents, uses substantially less sample than required for mouse bioassay, and is well within the scope of even modest tissue culture facilities. This cell-based protocol has the potential to serve as an alternate and complementary method to the standard mouse bioassay.

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Monitoring programs for marine toxins have depended in large part upon mouse bioassays. Although mouse assays have for many years provided a fairly reliable assessment of risk, there is mounting pressure to develop alternative methods to reduce the reliance on animal testing. Kogure et al. (1988) and Jellett et al. (1992) developed tissue culture assays in which mouse neuroblastoma cells are treated with veratridine and ouabain resulting in altered cell morphology and decrease in viability. Toxins which block sodium channels antagonize this effect, rescuing the cells in a dose dependent manner. Evaluation is either through the visual scoring of 200 or more cells per sample or well, a potentially time consuming and operator dependent task, or is dependent upon the physical removal of affected cells through the cumulative steps of rinsing, fixing, and staining.

We have developed a cell bioassay for detection and quantitation of sodium channel activating toxins such as the brevetoxins and ciguatoxins. We have modified and simplified the above assays for determination of sodium channel blockade. Assessment of cytotoxicity in the present method uses a colorimetric index of metabolic activity. Those cells which are metabolically active reduce a tetrazolium compound, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium), to a blue-colored formazan product (Mosmann,1983)). This method requires minimal processing and the results can be read on a standard multiwell scanning spectrophotometer (ELISA plate reader).

MATERIALS AND METHODS

TOXINS

Purified saxitoxin (Calbiochem) was diluted to the appropriate concentration with complete tissue culture medium prior to cell assay. Brevetoxins PbTx-1 and PbTx-3 (Calbiochem) were dissolved in methanol and diluted 1:100 in complete tissue culture medium, from which serial dilutions in complete medium were made. A ciguatoxic fish extract (methanol fraction), prepared from wrasse (*Cheilinus rhodochrous*) and mouse bioassay data were generously provided by Dr. Yoshitsugi Hokama, University of Hawaii. A stock solution of this material was prepared in the same manner as for the brevetoxins. Extracts of crab viscera and mouse bioassay data were generously made available by Cheryl Eklund and James Bryant, FDA, Bothell, WA.

MTT BIOASSAY

Cultures were prepared for bioassay as described by Jellett et al. (1992) with modifications described below. Neuro-2a cells (ATCC, CCL131) were grown in RPMI 1640 (Sigma) containing 10% fetal bovine serum (Gibco).

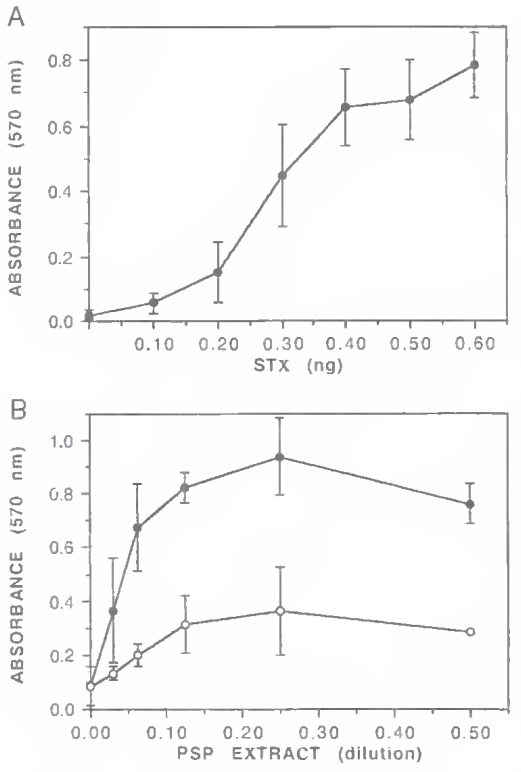

FIG.1.Effect of increasing concentration of pure saxitoxin upon MTT development in the neuroblastoma assay (1a). Standard MTT development time of approximately 15min allowed detection in the range of 0.1ng/10µl to 0.6ng/10µl saxitoxin addition per culture well. Aqueous extracts of Dungeness crab viscera examined for PSP activity in the MTT neuroblastoma assay (1b). Extracts that had tested at 122µg/100g (●) and no detectable activity (○) using the mouse bioassay were tested at various dilutions in the cell bioassay. Circle without error bar is the average of 2 replicates. The error bar indicates ±SD.Values represent mean of 3-4 replicates.

glutamine (2mM) (Sigma), sodium pyruvate (1mM) (Sigma), streptomycin 50µg/ml (Sigma), and penicillin 50units/ml (Sigma) (complete growth medium). Cells were seeded into 96-well plates at a density of 5 x 10^5 cells/ml in 200µl complete growth medium per well. Cultures were incubated at 37°C/5% CO_2 for approximately 24hr.

Culture wells received 10µl of sample and 10µl additions of aqueous stocks of 10mM ouabain (Sigma) and 1mM veratridine (Sigma) pH 2. Each sample concentration was tested in replicate (3 to 5 wells). A minimum of 15 wells per plate were processed as ouabain/veratridine treated controls (no sample addition), and a minimum of 5 wells served as untreated controls (without ouabain/veratridine and without sample). In the case of sodium channel activators, such as the brevetoxins and ciguatoxic extract, 10µl samples were added to replicate culture wells in both the presence and absence of ouabain and veratridine. Control wells received added culture medium to make up for volume differences.

Following incubation with samples, the overlaying medium was removed from cultures, and without a wash step, 60µl of a 1:6 dilution of MTT stock (5mg/ml in PBS, pH 7.4) in complete growth medium was added to each well. Cultures were incubated for approximately 15min at 37°C, medium was then removed, and 100µl of DMSO was added to each well. The plates were immediately read with an automated multiwell scanning spectrophotometer using a test wavelength of 570nm and a reference wavelength of 630nm.

RESULTS

Saxitoxin dependent inhibition of the ouabain/veratridine induced cytotoxicity was measured directly by alterations in MTT metabolism (Fig.1a). Purified saxitoxin was detected at a level of 0.1ng/10µl addition using an approximate MTT development time of 15min. Assay sensitivity could occasionally be enhanced by increasing MTT development time to c.45min, with a resultant detection limit of c.0.02ng/10µl addition (data not shown). Assay sensitivities were comparable to that reported by Jellett et al. (1992). In the absence of ouabain/veratridine treatment saxitoxin at the concentrations tested had no measurable effect. For the purpose of comparison, 0.1ng/10µl and 0.02ng/10µl saxitoxin are equivalent to shellfish extracts of 2µg/100g tissue and 0.4µg/100g respectively.

As a preliminary test of detection of naturally incurred PSP in samples, acid extracts of Dungeness crab viscera exhibiting positive and negative PSP activity by the AOAC mouse bioassay (122µg/100g and none detected/100g respectively) were examined by the MTT neuroblastoma assay (Fig.1b). The cell bioassay detected mean values (with standard deviations) of 124±44µg/100g in the positive extract (mouse bioassay) and 33±2µg/100g in the extract negative by mouse bioassay. The dose response curves tended to plateau with increasing concentrations of extract (dilutions of <1:4), suggesting a competing or potentially interfering cytotoxic component.

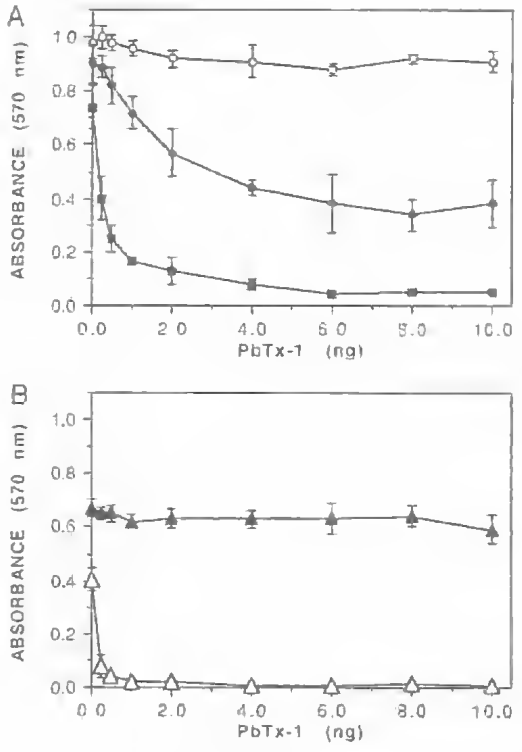

FIG.2. Brevetoxin PbTx-1 cytotoxicity as measured by the MTT neuroblastoma assay. (a, b) PbTx-1 cytotoxicity was assayed at 2hr (○), 4hr (●), 6hr (■), and 18hr (Δ). (b) Cytotoxicity was insignificant in the absence of ouabain and veratridine at the maximum incubation time of 18hr (▲). Values represent mean of 4 replicates. The error bar indicates ±SD.

Brevetoxins and ciguatoxins significantly enhance veratridine induced sodium influx in neuroblastoma cells (Catterall & Risk,1980; Bidard et al.,1984). We reasoned that this effect would accelerate the rate of ouabain/veratridine induced cytotoxicity and could therefore be the basis of a detection method for sodium channel activators such as brevetoxins and ciguatoxins. In the dose range explored, titratable cytotoxicity was observed as early as 4hr (Fig.2a) and was total at 18hr. Brevetoxin in the absence of ouabain/veratridine was not cytotoxic even at the highest concentration and incubation time tested (10ng/10µl, 18hr exposure) (Fig.2b). PbTx-3 produced similar results as observed for PbTx-1 in the cell bioassay (data not shown).

A ciguatoxic extract was examined with the same MTT cell assay format as utilized for brevetoxins. The extract was diluted and applied to neuroblastoma cells in the presence or absence of ouabain/veratridine. Within 6hr the sample produced significant dose dependent cytotoxicity only in cells treated with ouabain/veratridine (Fig.3). Even after prolonged exposures of up to 22hr the ciguatoxic extract was not cytotoxic in the MTT cell bioassay in the absence of ouabain/veratridine treatment (data not shown).

DISCUSSION

In the present study we sought to develop a diagnostic cell-based assay for determining either sodium channel blocking or enhancing activity. Furthermore, we explored the possibilities of improving previous methods by simplifying the end-point assessment of cells treated with sodium channel blocking agents in the presence of ouabain and veratridine.

Simplifying the assay was met by incorporating a sensitive colorimetric test of cellular metabolism, MTT, originally described by Mossman (1983). The method is a rapid, versatile, quantitative, and simple technique based upon the metabolism of a tetrazolium salt, MTT, by mitochondrial dehydrogenase activity in viable cells. This assay does not require washing or fixation steps. MTT metabolism has established itself as an accepted *in vitro* method in such diverse areas as the assessment of growth factor activity (Kotnik & Fleischmann,1990), radiosensitivity of cultured cells (Wasserman & Twentyman,1988), and the evaluation of chemotherapeutic agents upon target cell lines in culture (Carmichael et al.,

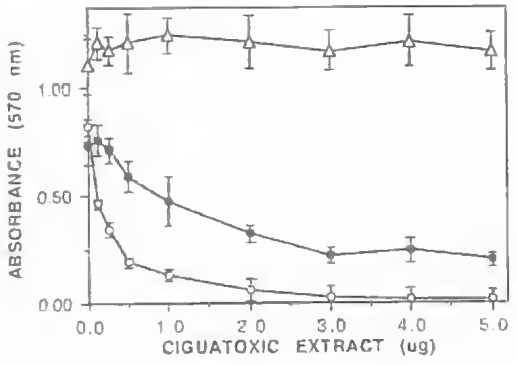

FIG.3. Ciguatoxic extract from wrasse analyzed by the MTT neuroblastoma assay. Ciguatoxic extract was diluted and applied to cells in the presence of ouabain and veratridine, 6hr (●) and 22hr (○), or without ouabain and veratridine, 22hr (Δ). Values represent mean of 4 replicates. The error bars indicate ±SD.

1987; Alley et al.,1988; Manger et al.,1989). Due to its many advantages and wide acceptance it seemed reasonable to explore the utility of MTT in a modified cell bioassay for the detection of marine toxins active at the sodium channel, and had been earlier suggested by us as a potentially useful approach (Manger,1993).

An additional goal of our studies was to incorporate modifications in the cell-bioassay to allow detection and quantitation of marine toxins that activate sodium channels. Insight as to how this might be accomplished came from Catterall & Risk (1980) and Bidard et al. (1984). Their research demonstrated that these toxins enhanced the ^{22}Na influx effect produced by veratridine treatment in neuroblastoma cells. We reasoned that this brevetoxin or ciguatoxin activity would also relate directly to an observable enhancement of ouabain/veratridine induced cytotoxicity in our assay. This was observed in our modified MTT neuroblastoma assay as exhibited by a dose dependent enhancement of cytotoxicity following treatment with either of these toxins. The lack of noticeable cytotoxicity in the absence of ouabain/veratridine is in agreement with specific toxin activity via interaction with sodium channels.

The MTT cell bioassay was significantly more sensitive than the mouse bioassay. The animal assay can detect saxitoxin to a lower limit of 40µg/100g tissue (Hungerford & Wekell,1992). In contrast, the cell bioassay can routinely detect purified saxitoxin at a level of 0.1ng/10ul, which is the equivalent of 2µg/100g tissue. Occasionally, with extended MTT development time, the observed limit of detection was 0.02µg/10µl (0.4µg/100g).

Examination of crab viscera samples with the MTT cell bioassay demonstrated good correlation with mouse bioassay results. A sample determined to have 122µg/100g tissue of PSP by mouse bioassay resulted in a mean value of 124±44µg/100g tissue using the MTT cell bioassay. Interestingly, a crab viscera sample that was PSP negative by mouse bioassay had a mean value of 33±2µg/100g tissue in the cell bioassay, however, this level of saxitoxin is below the standard detection limit of the animal test (40µg/100g). Examination of additional crab viscera extracts by MTT cell bioassay have produced results in agreement with the mouse bioassay (data not shown).

The modified MTT cell bioassay is also more sensitive to the brevetoxins than the mouse bioassay. The LD_{50} for mice is 0.01mg/20g animal, i.p. injection (Hungerford & Wekell,1992). This would correlate to 0.1mg/100g tissue extract and would be the equivalent of a 1ng/10µl sample in the MTT cell bioassay. In the present study the MTT cell bioassay detected brevetoxins at levels of 0.25ng/10µl.

The ciguatoxic extract tested in our studies produced death in 20g mice following injection of 50mg in 1ml within 2.5hr (Amra et al.,1990). This represents about 1.8 mouse units or the equivalent of 15ng CTX-1 as estimated by the method of Legrand et al. (1989). Thus, the sodium channel activity of this extract was readily detected in the MTT cell bioassay at levels of less than 10^{-4} mouse units, corresponding to low or sub pg concentrations of CTX-1.

Mouse bioassays for brevetoxins and ciguatoxins involve long observation periods, ranging from several hours to 48hr (Hungerford,1992), whereas, the MTT cell bioassay can be processed within 4-6hr. The MTT cell bioassay is also well suited to automation, providing a convenient biological assay that can accommodate a large number of samples and which can be accomplished within one day. Although *in vitro* methods cannot presently substitute entirely for the data derived from animal studies, these methods do offer the potential to reduce the reliance upon animal testing and to facilitate the rapid screening of test samples. In the event that mouse bioassays are prohibited or limited by law, cell bioassays for these marine toxins may provide an alternative screening method.

Subsequent to these preliminary studies we have confirmed the estimated assay sensitivity to ciguatoxins using purified CTX-1 and CTX3C (Manger et al., in press).

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